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(19)(CA) APPLICATION FOR CANADIAN PATENT (12)

(54) Anionic-Rich, High pH Liquid Detergent Compositions Containing Subtilisin Mutants

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ABSTRACT OF THE DISCLOSURE

The subject invention relates to mutant subtilisin proteases having substitution in at least 1 amino acid residue and to their use in anionic-rich, high-pH detergent compositions in view of the enhanced protease stability they provide.

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ANIONIC-RICH, HIGH-pH LIQUID DETERGENT COMPOSITIONS CONTAINING SUBTILISIN MUTANTS

BACKGROUND AND PRIOR ART

This invention relates to high-anionic, high-pH liquid detergent compositions containing mutant protease enzymes which provide enhanced stability.

The modification of subtilisin proteases by substitution at an amino acid site is known in the art. US-A-4 760 025, assigned to Genencor, for example, claims subtilisin mutants with amino acid substitutions at amino acid sites 32, 155, 104, 222, 166, 64, 33, 169, 217 or 157 which are different from subtilisins naturally produced by B. amyloliquefaciens. These amino acid substitutions are said to lead to increased oxidation stability of the protease.

WO 87/04461, assigned to Amgen, discloses the substitution in Bacillus subtilisins of alternative amino acids (i.e. serine, valine, threonine, cysteine, glutamine and isoleucine) for ASN, GLY or ASN-GLY sequences (specifically at position 218). These mutations are said to increase the stability of the enzyme at high temperatures or over a broader pH range than the wild type enzyme. WO 88/08033, also to Amgen, claims mutations which modify calcium-binding capacity (to replace an amino acid with a negatively charged residue such as ASP or GLU) and optionally a deletion and/or replacement of either residue of ASN-GLY sequences which results in better pH and thermal stability and higher specific activities. The reference claims that sites 41, 75, 76, 77, 78, 79, 80, 81, 208, and 214 may be replaced by a negatively charged amino acid and ASN may be replaced by SER, VAL, THR, CYS, GLU, or ILE in ASN-GLY sequences.

EP-A-342 177 (Procter & Gamble) discloses compositions comprising a protease with a specific mutation and having a pH between 7.0 and 9.0.

These references do not disclose anionic-rich, high-pH detergent compositions comprising the subtilisin mutants of the subject invention or the advantages provided by the use of these mutants in these detergent compositions.

WO 89/09819 (corresponding to US-A-4 980 288), assigned to Genex, discloses the subtilisin mutants which are used in the liquid detergent compositions of the invention. Although the use of mutants in washing preparations is disclosed (Claims 6 and 7), there is no teaching of the use of these mutants in anionic-rich, high-pH compositions. In particular, there is no disclosure of the use of these mutants in specific detergent compositions and no teaching or disclosure that the mutant enzymes will have enhanced stability in these specifically defined compositions.

SUMMARY OF THE INVENTION

The subject invention provides liquid detergent compositions comprising:

(1) from 5% to 65% by weight anionic surfactant or anionic surfactant and one or more detergent-actives wherein the ratio of anionic to non-anionic is greater than 1:1;

(2) from 0% to 50% by weight builder;

(3) a mutant subtilisin protease added in sufficient quantity to have an activity level of 0.01 to 100,000 GU/g having substitutions in 1 or more amino acid residues compared to wild type subtilisin or commercially available subtilisin; and

(4) remainder water and minor ingredients.

The pH of these compositions ranges from 9 to 12, preferably from 9.5 to 11.

According to the invention, when certain modified mutant subtilisin proteases are used in the above-identified anionic-rich, high-pH detergent compositions of the invention, enhanced stability is observed.

DETAILED DESCRIPTION OF THE INVENTION

Detergent-Active

The compositions of the invention comprise from about 5% to about 65% by weight of anionic surfactant or anionic surfactant and one or more detergent-actives wherein the ratio of anionic to non-anionic is greater than 1:1. Preferably, the compositions of the invention may comprise from 5-25% anionic and preferably from 10-2·% anionic; and from 5-15% preferably from 7-10% nonionic surfactant.

The detergent-active material other than anionic surfactant may be an alkali metal or alkanolamine soap or a 10 to 24 carbon atom fatty acid, including polymerized fatty acids, or a nonionic, cationic, zwitterionic or amphoteric synthetic detergent material, or mixtures of any of these.

Examples of the anionic synthetic detergents are salts (including sodium, potassium, ammonium and substituted ammonium salts) such as mono-, di- and triethanolamine salts of 9 to 20 carbon alkylbenzenesulphonates, 8 to 22 carbon primary or secondary alkanesulphonates, 8 to 24 carbon olefinsulphonates, sulphonated polycarboxylic acids prepared by sulphonation of the pyrolyzed product of alkaline earth metal citrates, e.g., as described in GB-A-1 082 179, 8 to 22 carbon alkylsulphates, 8 to 24 carbon alkylpolyglycol-ether-sulphates, -carboxylates and -phosphates (containing up to 10 moles of ethylene oxide); further examples are described in "Surface Active Agents and Detergents" (Vol. I and II) by Schwartz, Perry and Berch. Any suitable anionic may be used and the examples are not intended to be limiting in any way.

Examples of nonionic synthetic detergents which may be used with the invention are the condensation products of ethylene oxide, propyl ne oxide and/or butylene oxide with 8 to 18 carbon alkylphenols, 8 to ;8 carbon primary or secondary aliphatic alcohols, 8 to 18 carbon fatty acid amides; further

examples of nonionics include tertiary amine oxides with 8 to 18 carbon alkyl chain and two 1 to 3 carbon alkyl chains. The above reference also describes further examples of nonionics. The average number of moles of ethylene oxide and/or propylene oxide present in the above nonionics varies from 1-30; mixtures of various nonionics, including mixtures of nonionics with a lower and a higher degree of alkoxylation, may also be used.

Examples of cationic detergents which may be used are the quaternary ammonium compounds such as alkyldimethyl ammonium halogenides.

Examples of amphoteric or zwitterionic detergents which may be used with the invention are N-alkylamino acids, sulphobetaines, condensation products of fatty acids with protein hydrolysates; but, owing to their relatively high costs, they are usually used in combination with an anionic or a nonionic detergent. Mixtures of the various types of active detergents may also be used, and preference is given to mixtures of an anionic and a nonionic detergent active. Soaps (in the form of their sodium, potassium and substituted ammonium salts) of fatty acids may also be used, preferably in conjunction with an anionic and/or nonionic synthetic detergent.

Builders

Builders which can be used according to this invention include conventional alkaline detergency builders, inorganic or organic, which can be used at levels from 0% to about 50% by weight of the composition, preferably from 1% to about 20% by weight, most preferably from 2% to about 8%.

Examples of suitable inorganic alkaline det rgency builders are water-soluble alkalimetal phosphates, polyphosphates, borat s, silicates and also carbonates. Specific examples of such salts are sodium and potassium triphosphates,

pyrophosphates, orthophosphates, hexametaphosphates, tetraborates, silicates and carbonates.

Examples of suitable organic alkaline detergency builder salts are: (1) water-soluble amino polycarboxylates, e.g. sodium and potassium ethylenediaminetetraacetates, nitrilotriacetates and N-(2 hydroxyethyl)-nitrilodiacetates; (2) water-soluble salts of phytic acid, e.g. sodium and potassium phytates (see US-A-2 379 942); (3) water-soluble polyphosphonates, including specifically, sodium, potassium and lithium salts of ethane-1-hydroxy- 1,1-diphosphonic acid; sodium, potassium and lithium salts of methylene diphosphonic acid; sodium, potassium and lithium salts of ethylene diphosphonic acid; and sodium, potassium and lithium salts of ethane-1,1,2- triphosphonic acid. Other examples include the alkali metal salts of ethane-2-carboxy-1,1-diphosphonic acid hydroxymethanediphosphonic acid, carboxyldiphosphonic acid, ethane-1-hydroxy-1,1,2-triphosphonic acid, ethane-2-hydroxy-1,1,2-triphosphonic acid, propane- 1,1,3,3-tetraphosphonic acid, propane-1,1,2,3- tetraphosphonic acid, and propane-1,2,2,3- tetraphosphonic acid; (4) water-soluble salts of polycarboxylate polymers and co-polymers as described in US-A-3 308 067.

In addition, polycarboxylate builders can be used satisfactorily, including water-soluble salts of mellitic acid, citric acid, and carboxymethyloxysuccinic acid and salts of polymers of itaconic acid and maleic acid. Certain zeolites or aluminosilicates can be used. One such aluminosilicate which is useful in the compositions of the invention is an amorphous water-insoluble hydrated compound of the formula $Na_x(_yAlO_2.SiO_2)$, wh rein x is a number from 1.0 to 1.2 and y is 1, said amorphous mat rial being furth r characterized by an Mg^{++} exchange capacity of from about 50 mg eq. $CaCO_3/g$ and a particle diameter of from about 0.01 micron to about 5 microns. This ion- xchange builder is mor fully describ d in GB-A-1 470 250.

A second water-insoluble synthetic aluminosilicate ion-exchange material useful herein is crystalline in nature and has the formula $Na_z[(AlO_2)_y.(SiO_2)]xH_2O$, wherein z and y are integers of a least 6; the molar ratio of z to y is in the range from 1.0 to about 0.5, and x is an integer from about 15 to about 264; said aluminosilicate ion-exchange material having a particle size diameter from about 0.1 micron to about 100 microns; a calcium ion-exchange capacity on an anhydrous basis of at least about 200 milligrams equivalent of $CaCO_3$ hardness per gram; and a calcium-exchange rate on an anhydrous basis of at least about 2 grams/gallon/minute/gram. These synthetic aluminosilicates are more fully described in GB-A-1 429 143.

Mutant Subtilisin Protease

Proteins exist in a dynamic equilibrium between a folded, ordered state and an unfolded, disordered state. This equilibrium in part reflects the short range interactions between the different segments of the polypeptide chain which tend to stabilize the protein's structure, and, on the other hand, those thermodynamic forces which tend to promote the randomization of the molecule.

The largest class of naturally occurring proteins is made up of enzymes. Each enzyme generally catalyses a different kind of chemical reaction, and is usually highly specific in its function. Enzymes have been studied to determine correlations between the three-dimensional structure of the enzyme and its activity or stability.

The amino acid sequence of an enzyme determines the characteristics of the enzyme, and the enzyme's amino acid sequence is specified by the nucleotide sequence of a gene coding for the enzyme. A change of the amino acid sequence of an enzyme may alter the enzyme's properties to varying degrees, or may even inactivate the enzyme, depending on the

location, nature and/or magnitude of the change in the amino acid sequence.

Although there may be slight variations in a distinct type of naturally occurring enzyme within a given species or organism, enzymes of a specific type produced by organisms of the same species generally are substantially identical with respect to substrate specificity, thermal stability, activity levels under various conditions(e.g. temperature and pH), oxidation stability, and the like. Such characteristics of a naturally occurring or "wild-type" enzyme are not necessarily optimized for utilization outside of the natural environment of the enzyme. It may thus be desirable to alter a natural characteristic of an enzyme to optimize a certain property of the enzyme for a specific use, or for use in a specific environment.

Amino acids are naturally occurring compounds that are the building blocks of proteins. The natural amino acids are usually abbreviated to either three letters or one letter. The most common amino acids, and their symbols, are given in Table 1. The amino acids are joined head to tail to form a long main chain. Each kind of amino acid has a different side group.

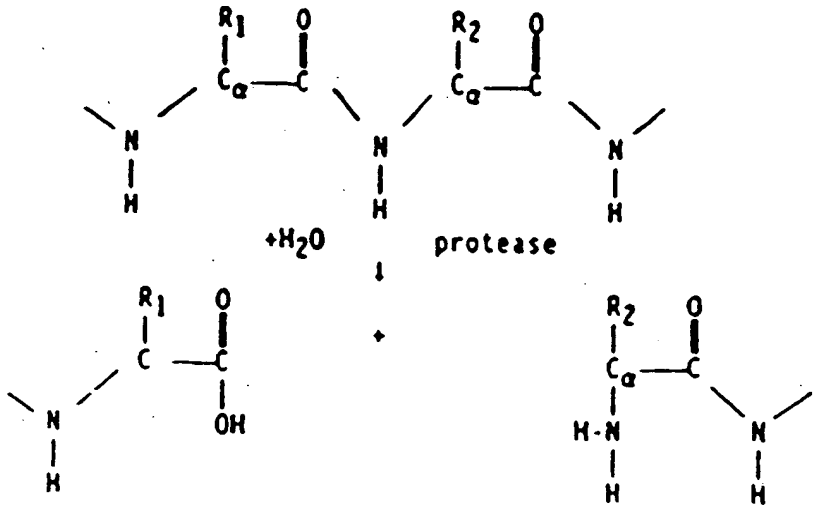
Table 1. Amino acid names and abbreviations

Amino acid	Three letter code	Single letter code
Alanine	Ala	A
Arginine	Arg	R
Aspartic acid	Asp	D
Asparagine	Asn	N
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

All amino acids have the same atoms in the main chain and differ only in the side chains. The main-chain atoms are a nitrogen, two carbons, and one oxygen. The first atom is the nitrogen, called N. The next atom is a carbon and is called the alpha-carbon. Side groups are attached to this alpha-carbon. The alpha-carbon is connected to the carbonyl carbon which is called C. C is connected to the carbonyl oxygen (called O) and to the N of the next residue. The side group atoms are given names composed of the symbol for the element (C, O, N, S), a Greek letter (alpha, beta, gamma, delta, epsilon, zeta and eta), and perhaps an Arabic numeral if the side group is forked.

The subtilisin enzymes used in the detergent compositions of this invention have been modified by mutating the various nucleotide sequences that code for the enzymes. Use of the modified subtilisin enzymes provides enhanced stability in the compositions.

The subtilisin enzymes of this invention belong to a class of enzymes known as proteases. A protease is a catalyst for the cleavage of peptide bonds. An example of this cleavage is given below:



One type of protease is a serine protease. A serine protease will catalyse the hydrolysis of peptide bonds in which there is an essential serine residue at the active site. Serine proteases can be inhibited by phenylmethyl sulphonylfluoride and by diisopropylfluoro phosphate.

A subtilisin is a serine protease produced by Gram positive bacteria or by fungi. The amino acid sequences of seven subtilisins are known. These include five subtilisins from Bacillus strains (subtilisin BPN', subtilisin Carlsberg, subtilisin DY, subtilisin amylosacchariticus, and mesenticopeptidase). (Vasantha et al., "Gene for alkaline protease and neutral protease from Bacillus amyloliquefaciens contain a large open-reading frame between the regions coding for signal sequence and mature protein, "J. Bacteriol. 159:811-819 (1984); Jacobs et al., "Cloning sequencing and expression of subtilisin Carlsberg form Bacillus licheniformis, "Nucleic Acids Res. 13:8013-8926 (1985); Nedkov et al., "Determination of the complete amino acid sequence of subtilisin DY and its comparison with the primary structures of the subtilisin BPN', Carlsberg and amylosacchariticus, "Biol. Chem. Hoppe-Seyler 366:421-430 (1985); Kurihara et al., "Subtilisin amylosacchariticus," J. Biol. Chem. 247:5619-5631 (1972); and Svendsen et al., "Complete amino acid sequence of alkaline mesenterico-peptidase," FEBS Lett. 196:228-232 (1986)).

The amino acid sequence of the subtilisin thermitase from Thermoactinomyces vulgaris is also known (Meloun et al., "Complete primary structure of thermitase from Thermoactinomyc s vulgaris and its structural features related to the subtilisin-type proteases," FEBS Lett. 183:195-200 (1985)). The amino acid sequences from two fungal proteinases are known: Proteinase K from Tritirachium album (Jany et al., "proteinase K from Tritirachium album Limber," Biol. Chem. Hoppe-Seyler 366:485-492 (1985)) and thermomycolase from the thermophilic fungus, Malbranchea

pulchella (Gaucher et al., "Endopeptidases: Thermomycolin," Methods Enzymol. 45:415-433 (1976)).

These enzymes have been shown to be related to subtilisin BPN', not only through their primary sequences and enzymological properties, but also by comparison of x-ray crystallographic data. (McPhalen et al., "Crystal and molecular structure of the inhibitor eglin from leeches in complex with subtilisin Carlsberg," FEBS Lett. 188:55-58 (1985) and Pahler et al., "Three-dimensional structure of fungal proteinase K reveals similarity to bacterial subtilisin," EMBO J. 3:1311-1314 (1984).)

The mutated enzymes used in the compositions of the invention may be introduced into any serine protease which has at least 50% and preferably 80% amino acid sequence homology with the sequence referenced above for subtilisin BPN', subtilisin Carlsberg, subtilisin DY, subtilisin amylosacchariticus, mesenticopeptidase, thermitase, proteinase K, or thermomycolase, and therefore may be considered homologous.

Thus, the mutated subtilisin enzymes used in the detergent composition of this invention have at least one of the specific amino acid position substitutions shown in Table 2. In Table 2, the naturally occurring amino acid and position number is given first with the arrow to the right indicating the amino acid substitution. The mutations were made using subtilisin BPN'. However, as explained herein, these mutations can be introduced at analogous positions in other serine proteases using oligonucleotide-directed mutagenesis.

Table 2

Mutations in subtilisin BPN'

1	Val18 -> Ile
2	Thr22 -> Cys, Ser87 -> Cys
3	Thr22 -> Lys, Asn76 -> Asp
4	Met50 -> Phe
5	Ser53 -> Thr
6	Ser63 -> Asp, Tyr217 -> Lys
7	Asn76 -> Asp
8	Ser78 -> Asp
9	Tyr104 -> Val, Gly128 -> Ser
10	Ala116 -> Glu
11	Leu126 -> Ile
12	Gly131 -> Asp
13	Gly166 -> Ser
14	Gly169 -> Ala
15	Pro172 -> Asp
16	Pro172 -> Glu
17	Ser188 -> Pro
18	Gln206 -> Cys
19	Gln206 -> Tyr
20	Ala216 -> Cys, Gln206 -> Cys
21	Tyr217 -> Lys
22	Tyr217 -> Leu
23	Asn218 -> Asp
24	Gln206 -> Tyr
25	Ser248 -> Asp, Ser249 -> Arg
26	Thr254 -> Ala
27	Gln271 -> Glu

In general, stability of a mutated subtilisin in a given composition is expressed as the half life of the enzyme in hours at a given temperature, e.g. 37°C.

Table 3 shows the strain designation of the host cell secreting the mutated subtilisin enzymes.

Table 3

Mutated Subtilisin BPN' Enzymes

Strain	Mutation
GX7130	Wild type
GX7174	VAL8->ILE
GX7175	GLY169->ALA
GX7181	ASN218->ASP
	THR22->CYS
	SER87->CYS
GX7186	ASN218->SER
	THR22->CYS
	SER87->CYS
	GLY169->ALA
GX7195	TYR217->LYS
GX7199	THR22->CYS
	SER87->CYS
	GLY169->ALA
	PRO172->ASP
GX8303	MET50->PHE
GX8309	SER248->ASP
	SER249->ARG
GX8314	GLN206->CYS
GX8321	THR22->CYS
	SER87->CYS
	GLY169->ALA
	MET50->PHE
	TYR217->LYS
	ASN218->SER
GX8324	THR22->CYS
	SER87->CYS
	GLY169->ALA
	MET50->PHE
	TYR217->LYS
	ASN218->SER
	GLN206->CYS
GX8330	TYR217->LEU

GX8336	GLN206->TYR
GX8350	MET50->PHE
	GLY169->ALA
	GLN206->CYS
	TYR217->LYS
	ASN218->SER
	ASN76->ASP
GX8352	SER63->ASP
	TYR217->LYS
GX8354	GLN271->GLU
GX8363	THR22->LYS
	ASN76->ASP
GX8372	MET50->PHE
	GLY169->ALA
	GLN206->CYS
	TYR217->LYS
	ASN76->ASP
	SER78->ASP
	ASN218->SER
GX8376	TYR104->VAL
	GLY128->SER
GX7148	GLY131->ASP
GX7150	ASN218->SER
GX7164	ASN218->ASP
GX7178	SER188->PRO
GX7188	ALA116->GLU
GX7189	LEU126->ILE
GX8301	ASN218->SER
	GLY166->SER
GX8305	SER53->THR
GX8306	ASN218->SER
	THR254->ALA
GX8315	ASN218->SER
	GLY131->ASP
	THR254->ALA
GX7159	THR22->CYS
	SER87->CYS

GX8307	GLN206->CYS
	SER87->CYS
	ALA216->CYS
GX7172	PRO172->ASP
GX8312	PRO172->GLU
GX8347	ASN76->ASP
GX8364	SER78->ASP
GX8373	ASN218->ASP
	MET50->PHE
	GLY169->ALA
	GLN206->CYS
	TYR217->LYS
	ASN76->ASP
	SER78->ASP
GX8397	MET50->PHE
	ASN76->ASP
	GLY169->ALA
	GLN206->CYS
	ASN218->SER
GX8398	MET50->PHE
	ASN76->ASP
	GLN206->CYS
	TYR217->LYS
	ASN218->SER
GX8399	MET50->PHE
	ASN76->ASP
	ASN218->SER
	GLN206->CYS

The subtilisin enzyme mutations, shown in Tables 2 and 3, can be made on other proteases which are closely related, subtilisin Carlsberg for example. Closeness of relation is measured by comparison of amino acid sequences. There are many methods of aligning protein sequences, but the differences are only manifested when the degree of relatedness is quite small. The methods described in Atlas of Protein Sequence and Structure, Margaret O. Dayhoff editor,

Vol. 5, Supplement 2, 1976, National Biomedical Research Foundation, Georgetown University Medical Center, Washington, D.C., p. 3 ff., entitled SEARCH and ALIGN, define relatedness. As is well known in the art, related proteins can differ in number of amino acids as well as identity of each amino acid along the chain. That is, there can be deletions or insertions when two structures are aligned for maximum identity. For example, subtilisin Carlsberg has only 274 amino acids, while subtilisin BPN' has 275 amino acids. Aligning the two sequences shows that Carlsberg has no residue corresponding to ASN56 of subtilisin BPN'. Thus the amino acid sequence of Carlsberg would appear very different from BPN' unless a gap is recorded at location 56. Therefore an analogous substitution of position 218 of BPN' may be made at location 218 of subtilisin Carlsberg, provided that the residues in Carlsberg are numbered by homology to BPN'.

In general, one should not transfer mutations if either subtilisin has a gap at, or immediately adjacent to, the site of the mutation. Therefore, after aligning the amino acid sequences, those mutations at, or next to, gaps should be deleted from the list of desirable mutations and the mutation is not made. One can use this reasoning to transfer all of the thermostable mutations described herein to other homologous serine proteases.

In brief, in order to introduce the mutation(s) for the subtilisin, the gene coding for the desired subtilisin material generally is first isolated from its natural source and cloned in a cloning vector. Alternatively, mRNA which is transcribed from the gene of interest can be isolated from the source cell and converted into cDNA by reverse transcription for insertion into a cloning vector. A cloning vector can be a phage or plasmid, and generally includes a replicon for autonomous replication of the vector in a micro-organism independent of the genome of the micro-organism. A cloning vector advantageously includes one or more phenotypic

markers, such as DNA coding for antibiotic resistance, to aid in selection of micro-organisms transformed by the vector.

Procedures for insertion of DNA or cDNA into a vector for cloning purposes are well known in the art. These procedures generally include insertion of the gene coding for the subtilisin material into an opened restriction endonuclease site in the vector, and may involve addition of homopolymeric tails of deoxynucleotides to the ends of the gene and linking the gene to opened ends of a cloning vector having complementary homopolymeric tails. A subtilisin gene can then be mutated by oligonucleotide-directed mutagenesis. Oligonucleotide-directed mutagenesis, also called site-directed mutagenesis, is described in detail in Bryan et al., Proc. Natl. Acad. Sci. USA 83:3743-3745 (1986), incorporated herein by reference.

The protease used in these compositions is used in an amount sufficient to have an activity of 0.01 to 100,000 GU/g based on the final composition. A GU is a glycine unit, which is the amount of proteolytic enzyme which under standard incubation conditions produces an amount of terminal NH_2-groups equivalent to 1 microgramme/ml of glycine.

Water

Finally, except for the stabilizer and optional components described below, water comprises the remainder of the compositions. Generally, the amount of water will vary from 30-80% of the composition although this will depend on the amount of actives and the ingredients used.

Stabilizer

Another component which may be optionally used in the compositions of the invention is a stabilizer or stabilizer system. The improvements in stability of the invention can be demonstrated in systems with or without enzyme stabilization systems although it is preferred that such systems be used.

When present, th stabilizati n system compris s from about 0.1 to about 15% of the composition.

The enzyme stabilization systems may comprise calcium ion, boric acid, propylene glycol and/or short chain carboxylic acids. The composition preferably contains from about 0.01 to about 50, preferably from about 0.1 to about 30, more preferably from about 1 to about 20 millimoles of calcium ion per liter.

When calcium ion is used, the level of calcium ion should be selected so that there is always some minimum level available for the enzyme after allowing for complexation with builders, etc. in the composition. Any water-soluble calcium salt can be used as the source of calcium ion including calcium chloride, calcium formate, calcium acetate, and calcium propionate. A small amount of calcium ion, generally from 0.05 to about 2.5 millimoles per liter, is often also present in the composition due to calcium in the enzyme slurry and formula water.

Another enzyme stabilizer which may be used is propionic acid or a propionic acid salt capable of forming propionic acid. When used, the stabilizer may be used in an amount from about 0.1% to about 15% by weight of the composition.

Another preferred enzyme stabilizer is polyols containing only carbon, hydrogen and oxygen atoms. They preferably contain from 2 to 6 carbon atoms and from 2 to 6 hydroxy groups. Examples include propylene glycol (especially 1,2 propanediol which is preferred), ethylene glycol, glycerol, sorbitol, mannitol and glucose. The polyol generally r pr s nts from about 0.5% t about 15%, preferably from about 1.0% to about 8% by w ight of the composition.

The composition h rein may also optionally contain from about 0.25% to about 5%, most pr ferably from about 0.5% to about

3% by weight of boric acid. The boric acid may be, but is preferably not, formed by a compound capable of forming boric acid in the composition. Boric acid is preferred, although other compounds such as boric oxide, borax and other alkali metal borates (e.g. sodium ortho-, meta-, and pyroborate and sodium pentaborate) are suitable. Substituted boric acids (e.g. phenylboronic acid, butane boronic acid and p-bromo phenylboronic acid) can also be used instead of boric acid.

One especially preferred stabilization system is a polyol in combination with boric acid. Preferably, the weight ratio of polyol to boric acid added is at least 1, more preferably at least 1.3.

Optional Components

In addition to the ingredients described hereinbefore, the preferred compositions herein frequently contain a series of optional ingredients which are used for the known functionality in conventional levels. While the inventive compositions are premised on aqueous enzyme-containing detergent compositions, it is frequently desirable to use a phase regulant. This component, together with water, then constitutes the solvent matrix for the claimed liquid compositions. Suitable phase regulants are well known in liquid detergent technology and, for example, can be represented by hydrotropes such as salts of alkylaryl-sulfonates having up to 3 carbon atoms in the alkylgroup, e.g. sodium, potassium, ammonium and ethanolamine salts of xylene-, toluene-, ethyl benzene-, cumene-, and isopropylbenzene sulfonic acids. Alcohols may also be used as phase regulants. This phase regulant is frequently used in an amount from about 0.5% to about 20%, the sum of phase regulant and water is normally in the range from 35% to 65%.

The preferred compositions herein can contain a series of further optional ingredients which are mostly used in additive levels, usually below about 5%. Examples of the like

additives include: polyacids, suds regulants, opacifiers, antioxidants, bactericides, dyes, perfumes, brighteners and the like.

The beneficial utilization of the claimed compositions under various usage conditions can require the utilization of a suds regulant. While generally all detergent suds regulants can be utilized, preferred for use herein are alkylated polysiloxanes such as dimethylpolysiloxane, also frequently termed silicones. The silicones are frequently used in a level not exceeding 0.5%, most preferably between 0.01% and 0.2%.

It can also be desirable to utilize opacifiers inasmuch as they contribute to create a uniform appearance of the concentrated liquid detergent compositions. Examples of suitable opacifiers include: polystyrene commercially known as LYTRON 621 manufactured by MONSANTO CHEMICAL CORPORATION. The opacifiers are frequently used in an amount from 0.3% to 1.5%.

The compositions herein can also contain known antioxidants for their known utility, frequently radical scavengers, in the art established levels, i.e. 0.001% to 0.25% (by reference to total composition). These antioxidants are frequently introduced in conjunction with fatty acids.

The compositions of the invention may also contain other enzymes in addition to the proteases of the invention such as lipases, amylases and cellulases. When present, these enzymes may be used in an amount from about 0.01% to about 5% of the compositions.

In a preferred embodiment of the invention, the formulation contains ingredients in the following ratio:

Ingredients	% by Weight
Linear alkylbenzene sulphonate	8-12
Alcohol ethoxylate	6-10
Alcohol ethoxysulphate	4- 8
Builder	5-10
Sodium xylene sulphonate	1- 5
Monoethanolamine	1- 3
Triethanolamine	1- 3
Mutant protease enzyme	*
Calcium chloride dihydrate	0- 0.1
Minor ingredients	< 1.0
Water	balance to 100
pH	9.0-12.0

* as required to provide activity of 0.01 to 100,000 GU/g, based on final composition.

In an especially preferred embodiment of this aspect of the invention, the mutant protease used in the above-formulated composition is GX 8379.

In a second preferred embodiment of the invention, the formulation contains ingredients in the following ratio:

Ingredients	% by Weight
Linear alkylbenzene sulphonate	8-12
Alcohol ethoxylate	6-10
Alcohol ethoxysulphate	4- 8
Builder	3- 7
Sodium xylene sulphonate	1- 5
Triethanolamine	1- 5
Borax pentahydrate	1- 5
Propylene glycol	2- 6
Calcium chloride dihydrate	0.035
Mutant protease enzyme	*
Minor ingredients	< 1.0
Water	balance to 100

pH	9.0-12.0

* as required to provide activity of 0.01 to 100,000 GU/g, based on final composition.

In an especially preferred embodiment of this aspect of the invention, the mutant protease used in the above-formulated composition is GX 8379.

Product pH

The pH of the compositions of the invention is from about 9 to about 12, preferably 9.5 to 11, most preferably 9.5 to 10.5.

The following examples are intended to illustrate the invention and facilitate its understanding and are not meant to limit the invention in any way.

EXAMPLE 1

The stability of various wild-type subtilisins were compared to mutant subtilisin strain GX8397 (subtilisin with 5 amino acid mutations) in the following formulations without stabilizer and with builder:

Anionic-rich formulation A

	Wt.%
Linear alkylbenzene sulphonate	10.0
Alcohol ethoxylate	8.0
Alcohol ethoxysulphate	6.0
Sodium citrate	7.0
Sodium xylene sulphonate	3.0
Monoethanolamine	2.0
Triethanolamine	2.0
Mutant protease enzyme	*
Calcium chloride dihydrate	0.035
Minor ingredients	0.5

Water	balance to 100
pH	10

* as required to provide activity of 0.01 to 100,000 GU/g, based on final composition.

Enzyme	Half-life at 37°C (hrs)	% Improvement
Savinase (from Novo)	119	-
Alcalase (from Novo)	49	-
Wild type BPN' (from Novo)	89	-
GX 8397	440	269*

*Relative to Savinase; relative to Alcalase, improvement was 797% and relative to BPN', the improvement was 394%.

Anionic-rich formulation B

	Wt.%
Linear alkylbenzene sulphonate	10.0
Alcohol ethoxylate	8.0
Alcohol ethoxysulphate	6.0
Sodium citrate	5.0
Sodium xylene sulphonate	2.5
Triethanolamine	3.0
Borax pentahydrate	2.4
Propylene glycol	4.0
Calcium chloride dihydrate	0.035
Mutant protease enzyme	*
Minor ingredients	< 1.0
Water	balance to 100
pH	9.8

* as required to provide activity of 0.01 to 100,000 GU/g, based on final composition.

Enzyme	Half-life at 37°C (hrs)	% Improvement
Savinase (from Novo)	197	-
GX 8397	500	153

As can be seen from the results above, the stability of the mutant strain GX8397, measured as the half-life of the enzym at 37°C, was significantly greater in the anionic rich, high pH compositions of the invention compared to the wild-type and/or commercially available enzymes tested in the same formulations.

EXAMPLE 2

The stability of wild-type BPN' was compared to mutant subtilisins with 6 or fewer amino acid mutations in formulation A.

Enzyme	No. of Amino Acid Substitutions	Half-life at 37°C (hrs)
Wildtype BPN'	0	73
GX 8350	6	441
GX 8397	5	523
GX 8398	5	459
GX 8399	4	380
GX 7160	1	135
GX 7175	1	103
GX 7195	1	120
GX 8303	1	125
GX 8314	1	145
GX 8347	1	155

The results show that the stability of the mutant enzymes was clearly superior to wild-type BPN' in the composition of the invention. The example also shows that stability was significantly improved even when the enzyme was mutated in as few as 1 amino acid site.

EXAMPLE 3

The stability of Savinase enzyme was compared to GX 8350 (subtilisin with 6 amino acid mutations) in Formulation A with and without builder (i.e. 7.0% sodium citrate).

Formulation A	Half-life at 37°C (hrs) Savinase	GX 8350
with builder	123	441
without builder	246	410

The results show that the stability of GX 8350 is not significantly affected by builder, while the presence of a builder in formulation A has a major impact on Savinase stability.

EXAMPLE 4

The stability of various subtilisins were compared to GX 8350 (subtilisin with 6 amino acid substitutions) in Formulation A with varying amounts of enzyme stabilizer.

	Half-Life at 37°C (hours)		
Enzyme	No stabilizer	1/2 stabilizer	Full stabilizer
BPN'	77	190	427
Savinase	119	350	732
Alcalase	49	105	198
GX 8350	433	1022	2300

The stabilizer system used in the above examples was a propylene glycol/borax stabilizer system. The 1/2 stabilizer system comprises 2.12% propylene glycol and 1.33% borax (introduced as sodium borate tetrahydrate) and the full stabilizer system comprises 4% propylene glycol and 2.7% borax (also introduced as sodium borate tetrahydrate). All percentages were by weight.

These results show that the stability of enzymes tested is improved with the use of stabilizer (although use of the stabilizer is not required). The stability in formulation A was much greater, with or without stabilizer, when GX8350 enzyme was used.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A liquid detergent composition having a pH in the range of from about 9.0 to about 12.0 comprising the following:

Ingredients	% by Weight
Linear alkylbenzene sulphonate	8-12
Alcohol ethoxylate	6-10
Alcohol ethoxysulphate	4- 8
Builder	5-10
Sodium xylene sulphonate	1- 5
Monoethanolamine	1- 3
Triethanolamine	1- 3
Mutant protease enzyme	*
Calcium chloride dihydrate	0- 0.1
Minor ingredients	< 1.0
Water	balance to 100

* as required to provide activity of 0.01 to 100,000 GU/g, based on final composition.

2. A liquid detergent composition according to claim 1, wherein the subtilisin is derived from Strain GX8350 and has the following substitutions:

MET50->PHE
GLY169->ALA
GLN206->CYS
TYR217->LYS
ASN218->SER
ASN76->ASP

3. A liquid detergent composition according to claim 1, wherein the subtilisin is derived from Strain GX8397 and has the following mutations:

MET50->PHE
ASN76->ASP
GLY169->ALA

GLN206->CYS
ASN218->SER

4. A liquid detergent composition according to claim 1, wherein the subtilisin is derived from GX8398 and has the following mutations:

MET50->PHE
ASN76->ASP
GLN206->CYS
TYR217->LYS
ASN218->SER

5. A liquid detergent composition according to claim 1, wherein the subtilisin is derived from Strain GX8399 and has the following mutations:

MET50->PHE
ASN76->ASP
ASN218->SER
GLN206->CYS

6. A liquid detergent composition according to claim 1, comprising the following:

	Wt.%
Linear alkylbenzene sulphonate	10.0
Alcohol ethoxylate	8.0
Alcohol ethoxysulphate	6.0
Sodium citrate	7.0
Sodium xylene sulphonate	3.0
Monoethanolamine	2.0
Triethanolamine	2.0
Mutant protease enzyme	*
Calcium chloride dihydrate	0.035
Minor ingredients	0.5
Water	balance to 100
pH	10

7. A liquid detergent composition according to claim 6, wherein the mutant protease is GX 8397.

8. A liquid detergent composition according to claim 1, additionally comprising 0.5 to about 15% by weight of an enzyme stabilizer as enzyme stabilization system.

9. A composition according to claim 8, wherein the enzyme stabilizer is propionic acid or a propionic acid salt capable of forming propionic acid.

10. A composition according to claim 1, wherein the enzyme stabilizer is an enzyme stabilizer system comprising propylene glycol and boric acid.

11. A liquid detergent composition having a pH in the range of from about 9.0 to about 12.0 comprising the following:

Ingredients	% by Weight
Linear alkylbenzene sulphonate	8-12
Alcohol ethoxylate	6-10
Alcohol ethoxysulphate	4- 8
Builder	3- 7
Sodium xylene sulphonate	1- 5
Triethanolamine	1- 5
Borax pentahydrate	1- 5
Propylene glycol	2- 6
Calcium chloride dihydrate	0.035
Mutant protease enzyme	*
Minor ingredients	< 1.0
Water	balance to 100
pH	9.0-12.0

* as required to provide activity of 0.01 to 100,000 GU/g, based on final composition.

12. A liquid detergent composition according to claim 11, wherein the subtilisin is derived from Strain GX8350 and has the following substitutions:

MET50->PHE

GLY169->ALA

GLN206->CYS

TYR217->LYS
ASN218->SER
ASN76->ASP

13. A liquid detergent composition according to claim 11, wherein the subtilisin is derived from Strain GX8397 and has the following mutations:

MET50->PHE
ASN76->ASP
GLY169->ALA
GLN206->CYS
ASN218->SER

14. A liquid detergent composition according to claim 11, wherein the subtilisin is derived from GX8398 and has the following mutations:

MET50->PHE
ASN76->ASP
GLN206->CYS
TYR217->LYS
ASN218->SER

15. A liquid detergent composition according to claim 11, wherein the subtilisin is derived from Strain GX8399 and has the following mutations:

MET50->PHE
ASN76->ASP
ASN218->SER
GLN206->CYS

16. A liquid detergent composition according to claim 11, comprising the following:

	Wt.%
Linear alkylbenzene sulphonate	10.0
Alcohol ethoxylate	8.0
Alcohol ethoxysulphate	6.0
Sodium citrate	5.0
Sodium xylene sulphonate	2.5

Triethanolamine	3.0
Borax pentahydrate	2.4
Propylene glycol	4.0
Calcium chloride dihydrate	0.035
Mutant protease enzyme	*
Minor ingredients	< 1.0
Water	balance to 100
pH	9.8

* as required to provide activity of 0.01 to 100,000 GU/g, based on final composition.

17. A liquid detergent composition according to claim 16, wherein the mutant protease is GX 8397.

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18. A liquid detergent as claimed in claim 1 and substantially as described herein.